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Expression of cholera toxin B–proinsulin fusion protein in lettuce and tobacco chloroplasts – oral administration protects against development of insulitis in non-obese diabetic mice

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Summary

Lettuce and tobacco chloroplast transgenic lines expressing the cholera toxin B subunit–human proinsulin (CTB-Pins) fusion protein were generated. CTB-Pins accumulated up to ~16% of total soluble protein (TSP) in tobacco and up to ~2.5% of TSP in lettuce. Eight milligrams of powdered tobacco leaf material expressing CTB-Pins or, as negative controls, CTB–green fluorescent protein (CTB-GFP) or interferon–GFP (IFN-GFP), or untransformed leaf, were administered orally, each week for 7 weeks, to 5-week-old female non-obese diabetic (NOD) mice. The pancreas of CTB-Pins-treated mice showed decreased infiltration of cells characteristic of lymphocytes (insulitis); insulin-producing β-cells in the pancreatic islets of CTB-Pins-treated mice were significantly preserved, with lower blood or urine glucose levels, by contrast with the few β-cells remaining in the pancreatic islets of the negative controls. Increased expression of immunosuppressive cytokines, such as interleukin-4 and interleukin-10 (IL-4 and IL-10), was observed in the pancreas of CTB-Pins-treated NOD mice. Serum levels of immunoglobulin G1 (IgG1), but not IgG2a, were elevated in CTB-Pins-treated mice. Taken together, T-helper 2 (Th2) lymphocyte-mediated oral tolerance is a likely mechanism for the prevention of pancreatic insulitis and the preservation of insulin-producing β-cells. This is the first report of expression of a therapeutic protein in transgenic chloroplasts of an edible crop. Transplastomic lettuce plants expressing CTB-Pins grew normally and transgenes were maternally inherited in T_1 progeny. This opens up the possibility for the low-cost production and delivery of human therapeutic proteins, and a strategy for the treatment of various other autoimmune diseases.

Keywords: autoimmune therapy, diabetes, edible crop, oral tolerance, plant-made pharmaceuticals.

Introduction

Diabetes is a disease in which the body does not produce or properly utilize insulin. Type 1 diabetes results from the autoimmune destruction of insulin-producing cells. The major destruction of β-cells occurs predominantly from auto-reactive T-cytotoxic cells (Nagata *et al.*, 1994) and T-helper-1 (Th1) cells (Ploix *et al.*, 1999) reactive to β-cell autoantigens, such as insulin. In 2002, the American Diabetes Association estimated that 18.2 million people in the USA, or 6.3% of the total population, had diabetes, with more than $120 billion in treatment costs each year. In 2002, diabetes was the sixth leading cause of death in the USA, contributing to 213 062 deaths. The only currently accepted form of treatment is the administration of recombinant insulin, which serves to temporarily replace the missing insulin in diabetic patients. Therefore, it is essential to find a prevention and cure for this debilitating disease.

Biopharmaceutical proteins expressed in plant cells should reduce their cost of production. Transformation of plant nuclear genomes has led to the expression of a number of clinically important molecules in cell culture, organized tissue

culture and whole plants (Rigano and Walmsley, 2005). Common crop species, such as potatoes, rice and tomatoes, have been engineered to express many therapeutic proteins via the nuclear genomes of these plants (Ma *et al*., 2003). One of the major limitations has been the ability in these systems to accumulate sufficient levels of protein, either for purification or for oral delivery in minimally processed plant tissues. The integration of transgenes via the nuclear genome may have other disadvantages, including transgene containment, gene silencing and position effect. The chloroplast genetic engineering approach can overcome concerns about transgene containment (Daniell, 2002), gene silencing and position effect (De Cosa *et al*., 2001; Lee *et al*., 2003), pleiotropic effects (Daniell *et al*., 2001; Lee *et al*., 2003) and the presence of antibiotic-resistant genes or vector sequences in transformed genomes (Daniell *et al*., 2004a,b, 2005a,b; Grevich and Daniell, 2005). Multigene engineering is possible with chloroplast transformation because of its prokaryotic nature (De Cosa *et al*., 2001; Quesada-Vargas *et al*., 2005). Several vaccine antigens have been expressed via the chloroplast genome against bacterial, viral and protozoan pathogens, including the cholera toxin B subunit (CTB) (Daniell *et al*., 2001), anthrax protective antigen (Watson *et al*., 2004; Koya *et al*., 2005), the C-terminus of *Clostridium tetani* (Tregoning *et al*. 2003), the 2L21 peptide from the canine parvovirus (Molina *et al*., 2004), rotavirus VP6 protein (Birch-Machin *et al*., 2004) and the GAL/GALNAc lectin of *Entamoeba histolytica* (Chebolu and Daniell, 2007).

In addition to their use for the hyper-expression of vaccine antigens, transgenic chloroplasts have been employed in our laboratory for the production of valuable therapeutic proteins, such as human elastin-derived polymers for various biomedical applications (Guda *et al*., 2000), human serum albumin (Fernandez-San Millan *et al*., 2003), magainin (a broad-spectrum topical agent, systemic antibiotic, wound healing stimulant and potential anticancer agent) (DeGray *et al*., 2001), various interferon-α (IFN-α) proteins (Daniell *et al*., 2004a, 2005a; Arlen *et al*., 2007) and insulin-like growth factor 1 (Daniell *et al*., 2005a). Several other laboratories have expressed other therapeutic proteins, including human somatotropin (Staub *et al*., 2000) and IFN-γ–β-glucuronidase (IFN-γ-GUS) fusion proteins (Leelavathi and Reddy, 2003), in transgenic chloroplasts. The successful expression and assembly of complex multisubunit proteins, exemplified above, demonstrate that chloroplasts contain the machinery that allows for correct folding and disulphide bond formation, resulting in fully functional proteins (Kamarajugadda and Daniell, 2006).

The oral delivery of biopharmaceutical proteins expressed in plant cells should reduce the costs associated with purification, processing, cold storage, transportation and delivery. However, poor intestinal absorption of intact proteins has been a major challenge. To overcome this limitation, we investigated the concept of receptor-mediated oral delivery of transgenic proteins (Limaye *et al*., 2006). The B subunit of the toxin from *Vibrio cholerae* (CTB) is recognized as one of the most potent mucosal adjuvants (Holmgren *et al*., 2005). CTB and green fluorescent protein (CTB-GFP), separated by a furin cleavage site, were expressed via the tobacco chloroplast genome. Following the oral administration of CTB-GFP-expressing leaf material to mice, GFP was observed in the mice intestinal mucosa, liver and spleen in fluorescence and immunohistochemical studies, whereas CTB remained in the intestinal cell (Limaye *et al*., 2006). This report of receptor-mediated oral delivery of a foreign protein into the circulatory system brings the oral delivery of human therapeutic proteins one step closer to realization.

Clearly, there are advantages to plastid expression; however, in terms of minimally processed orally delivered vegetative biomass, there is a need to move beyond tobacco and to focus on edible crop plant systems. The transformation of plastids in non-green tissue has been achieved, especially in edible parts of carrot, providing access to several new potential platforms for pharmaceutical production (Kumar *et al*., 2004a,b; Daniell *et al*., 2005b). For this study, *Lactuca sativa* (lettuce) was chosen for investigation. Although, recently, two laboratories have reported stable plastid transformation in lettuce, the expression of therapeutic proteins has been unsuccessful (Lelivelt *et al*., 2005; Kanamoto *et al*., 2006).

The non-obese diabetic (NOD) mouse is a useful animal model for research in human diabetes (Homann *et al*., 1999). These mice show signs of insulitis as a result of lymphocytic infiltration of the endocrine part of the pancreas, which leads to decreased production of insulin and increased blood sugar, with its consequent pathologies. In this study, the effect of the oral administration of tobacco chloroplast-derived pro-insulin conjugated to CTB (CTB-Pins fusion) for the induction of oral tolerance towards insulin was examined. The first report of the accumulation of a valuable therapeutic protein, the CTB-Pins fusion, in lettuce chloroplasts is also presented.

Results

Vector construction of pLD-5′UTR-CTB-Pins

The *CTB-Pins* fusion gene was inserted into the chloroplast transformation vector pLD-ctv as described previously (Daniell *et al*., 2004b). The 5CP construct was expressed under the control of the *psb*A 5′ untranslated region (UTR)/promoter in

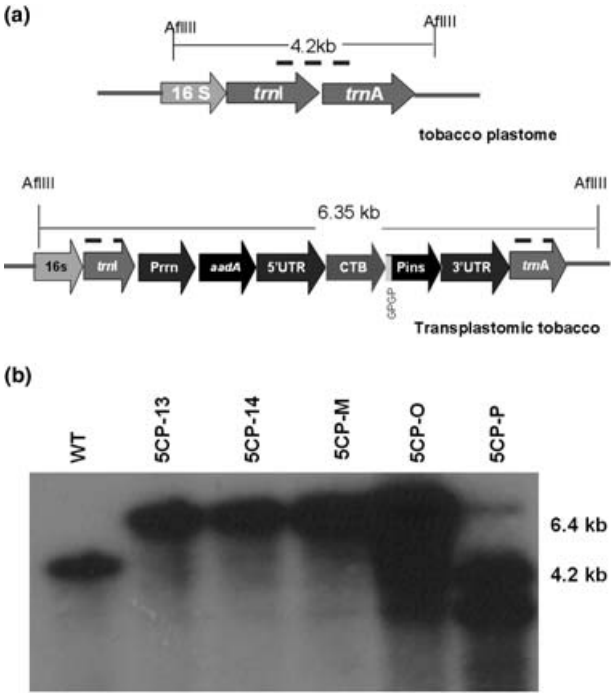

Figure 1 Southern analysis of cholera toxin B subunit–human proinsulin (CTB-Pins) tobacco transformants. (a) Schematic representation of expected results in Southern analysis. Full lines indicate expected fragment for wild-type (4.2 kb) and transformed (6.35 kb) genomes; broken line indicates the probe hybridization sites. (b) Southern analysis of second regenerants. 5CP lines 13, 14, M, O and P were derived from independent transformation events; WT, wild-type.

order to achieve hyper-expression, as demonstrated previously for other transgenes (Daniell *et al.*, 2004b). To prevent steric hindrance, a glycine–proline–glycine–proline (GPGP) hinge was introduced between the fusion elements (Arakawa *et al.*, 1998). The 3′ UTR located at the 3′ end of the introduced gene confers transcript stability (Stern and Gruissem, 1987; Figure 1a).

The *L. sativa* long flanking plastid transformation vector (pLS-LF, Figure 2a) was constructed using primers derived from regions of known conservation in the *Nicotiana tabacum* plastome to amplify cognate sequences from the *L. sativa* plastome. The full-length genes for plastid *trnI* (Ile) (1020 bp) and *trnA* (Ala) (887 bp) were amplified and cloned. The unique *Pvu*II site was used to insert transformation cassettes into the intergenic spacer region between the tRNAs. Long flanking sequences encoding portions of the 16S (810 bp) and 23S (974 bp) ribosomal RNA subunits were included to enhance homologous recombination between the vector and the host plastome. The tobacco native plastid ribosomal operon promoter (P*rrn*) and 3′ UTRs (*psbA* and *rps*16) were used to drive the expression of AadA from the GGAGG ribosome binding site and *CTB-Pins* from the 5′-translation control element of bacteriophage T7 gene10 (Figure 2a). Following the assembly of pLS-LF-CTB-Pins, plasmids were isolated from spectinomycin-resistant cultures and correct orientation was confirmed by *Sac*I digest.

Transformation and regeneration of transplastomic plants

The bombardment of 10 tobacco leaves resulted in 56 independent transformation events, 40 of which were subjected to three rounds of selection, two on regeneration medium of plants (RMOP) and one on Murashige and Skoog medium without hormones (MS0) medium containing 500 mg/L spectinomycin. Integration of the transgene cassette was confirmed by polymerase chain reaction (PCR).

The bombardment of 60 lettuce leaves resulted in two independent transformation events (lines L100 and L101), identified as green shoots arising directly from completely bleached tissue on lettuce regeneration (LR) medium containing 50 mg/L spectinomycin (Figure 2d). It should be noted that no callus was formed prior to the initiation of shoots from leaf explants. Following an additional round of selective regeneration, a progenitor for each line was rooted in MS medium containing spectinomycin (50 mg/L) and multiplied by clonal propagation. Clones were transferred to soil (Figure 3a) and moved to the glasshouse, where they matured (Figure 3b), bolted and produced normal panicles (Figure 3c) and inflorescences with disc flowers (Figure 3d). Pappus-bearing achenes (seeds) were harvested. Lettuce seeds (T_1) were sterilized and germinated on spectinomycin-containing medium (50 mg/L) together with wild-type (WT) seeds (Figure 3e). T_1 seeds germinated and grew into uniformly green plants (Figure 3f). The absence of Mendelian segregation of transgenes indicated that they were maternally inherited to progeny. Most WT seeds failed to germinate on spectinomycin-containing medium. Of the 30 WT seeds plated, six reached radicle emergence, but hypocotyl emergence was not observed and none developed into seedlings.

Analysis of the transgenic chloroplast genome confirms homoplasmy

Chloroplast transgenic lines were examined by Southern analysis in order to confirm site-specific integration and to determine whether they were homoplasmic or heteroplasmic. Homoplasmy is achieved when all the copies of the genome within the chloroplast have stably integrated transgenes. For tobacco, the gene-specific probe (*CTB-Pins*) taken from the

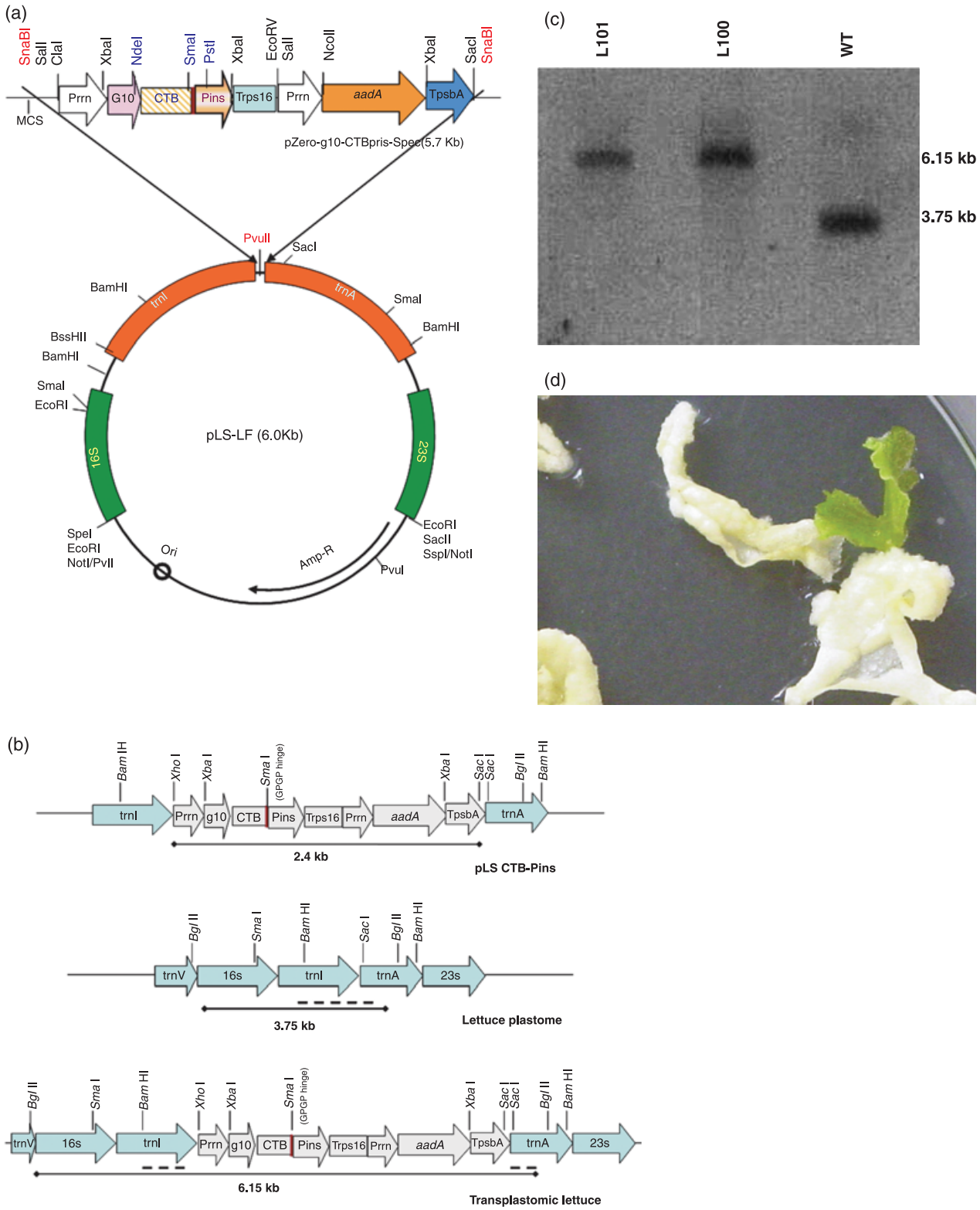

Figure 2 Regeneration and Southern analysis of cholera toxin B subunit–human proinsulin (CTB-Pins) lettuce transformants. (a, b) Schematic diagrams of lettuce transformation vector and integration cassette, integration site and resulting transplastome. Full lines indicate the sizes of the integration cassette (2.4 kb) and expected fragments for the wild-type (3.75 kb) and transformed (6.15 kb) genomes in Southern analysis; broken line indicates the probe hybridization sites. (c) Southern analysis of second regenerants. L100 and L101, independent transplastomic lines; WT, wild-type. (d) Primary regeneration in lettuce without formation of callus.

pLD-5CP vector by *Mfe*I/*Not*I digestion hybridized to a single 6.4-kb fragment in transgenics, but not to any WT plant DNA fragment (data not shown). The flanking sequence probe contained regions of the *trn*I and *trn*A genes. In most of the transgenic lines, only the 6.4-kb fragment was seen when hybridized with this probe (Figure 1b), indicating that homoplasmy was achieved within limits of detection by Southern blot.

For the confirmation of homoplasmy in lettuce transgenic lines, labelled flanking sequence probe (1.3 kb) was hybridized to *Bgl*II-digested genomic DNA. The hybridization pattern showed only the expected 3.75-kb fragment in the WT sample and only the expected 6.15-kb fragment in lettuce transformants after second regeneration (Figure 2c). Southern blotting was also carried out with genomic DNA samples from the primary regenerants. Although the L100 line demonstrated heteroplasmy, with both WT and transformed fragments hybridizing with the probe, primary regenerant L101 appeared to be homoplasmic (data not shown). Previous studies with tobacco have demonstrated that homoplasmy can indeed be achieved in the first round of selection (Guda *et al.*, 2000), because the flanking sequence contained an origin of replication (thereby providing more templates for integration). As the lettuce transformation vector includes full-length lettuce *trn*I gene, which probably contains *Ori*A, it is not unreasonable to find this phenomenon in lettuce plastid transformants as well.

CTB-Pins accumulation and pentamer assembly in transgenic chloroplasts

Immunoblots probed with the human proinsulin monoclonal antibody showed the presence of an ~22-kDa fusion protein in the chloroplast transgenic lines. The formation of dimers, trimers, tetramers and pentamers of the CTB-Pins fusion protein was also observed (Figure 4a,b). A similar banding pattern was observed by immunodetection with CTB polyclonal antibody (Figure 4c). Quantification of the fusion protein on western blots was performed by comparing plant samples with known quantities of purified CTB and analysing them by spot densitometry. Linearity of the standard curve was achieved using 25, 50 and 100 ng of CTB, facilitating the estimation of CTB expression in samples in the same blot (Figure 4d). The three tobacco transgenic lines were found to contain up to 178.2, 126.7 and 139.1 μg of CTB-Pins per 100 mg of leaf tissue, i.e. approximately 16% of total soluble protein (TSP) (Figure 4e). Such variation could be the result of the use of fresh vs. frozen plant material for these assays, or differences in sample preparation or growth conditions. Lettuce lines L100 and L101 accumulated up to 13.6 and 15.3 μg per 100 mg of leaf tissue, respectively, approximately 2.02% and 2.43% of TSP. The GM_1 binding assay demonstrated that pentameric structures of CTB-Pins were formed (Figure 4f). This assay confirmed the correct folding and disulphide bond formation of CTB pentamers within transgenic chloroplasts, as only the pentameric structure of CTB has the ability to bind to the GM_1 receptor (Merritt *et al.*, 1994).

Despite a number of experimental approaches, we were unable to establish the expression level of CTB-Pins in tobacco or lettuce plants via enzyme-linked immunosorbent assay (ELISA). The highest absorbance values were observed in the GM_1 binding assay from samples extracted under reducing conditions. Although we would expect reduction of disulphide bonds to abrogate the ability of our fusion protein to associate with the plate-bound receptor in pentameric form (Ludwig *et al.*, 1985; Dertzbaugh and Cox, 1998), plant extracts prepared in western extraction buffer [14 mM (beta)-mercaptoethanol (β-me)] or with a molar excess of dithiothreitol (0.5 M DTT) clearly facilitated GM_1 binding, as indicated by elevated absorbance readings. The potential for protein aggregation to occur in plant extracts increases with the concentration of the protein in question. Together, CTB monomers and the proinsulin molecule contain several cysteine residues through which disulphide bonds form, both intramolecularly and intermolecularly. We believe that the aggregation of the CTB-Pins fusion confounded our efforts to execute quantitative ELISAs.

Lymphocytic infiltration of the endocrine part of the pancreas (insulitis)

Insulitis is characterized by lymphocytic infiltration of the pancreatic islets, which leads to their destruction, including the insulin-producing β-cells. The pancreases were collected from 12-week-old NOD mice from the different treatment groups to assess the degree of insulitis.

To quantify and compare insulitis between the treatment groups, representative sections were prepared and cellular infiltration was scored in a blind manner. Fifty sections per animal were analysed. The average score indicated that the pancreases from NOD mice treated with CTB-Pins showed minimal cellular infiltration, and this cellular infiltration was significantly less than in all the other treatment groups (Figure 5a–c).

Preservation of insulin-producing β-cells following oral delivery of CTB-Pins

We next determined whether the remaining β-cells in the pancreases of the different treatment groups were apoptotic.

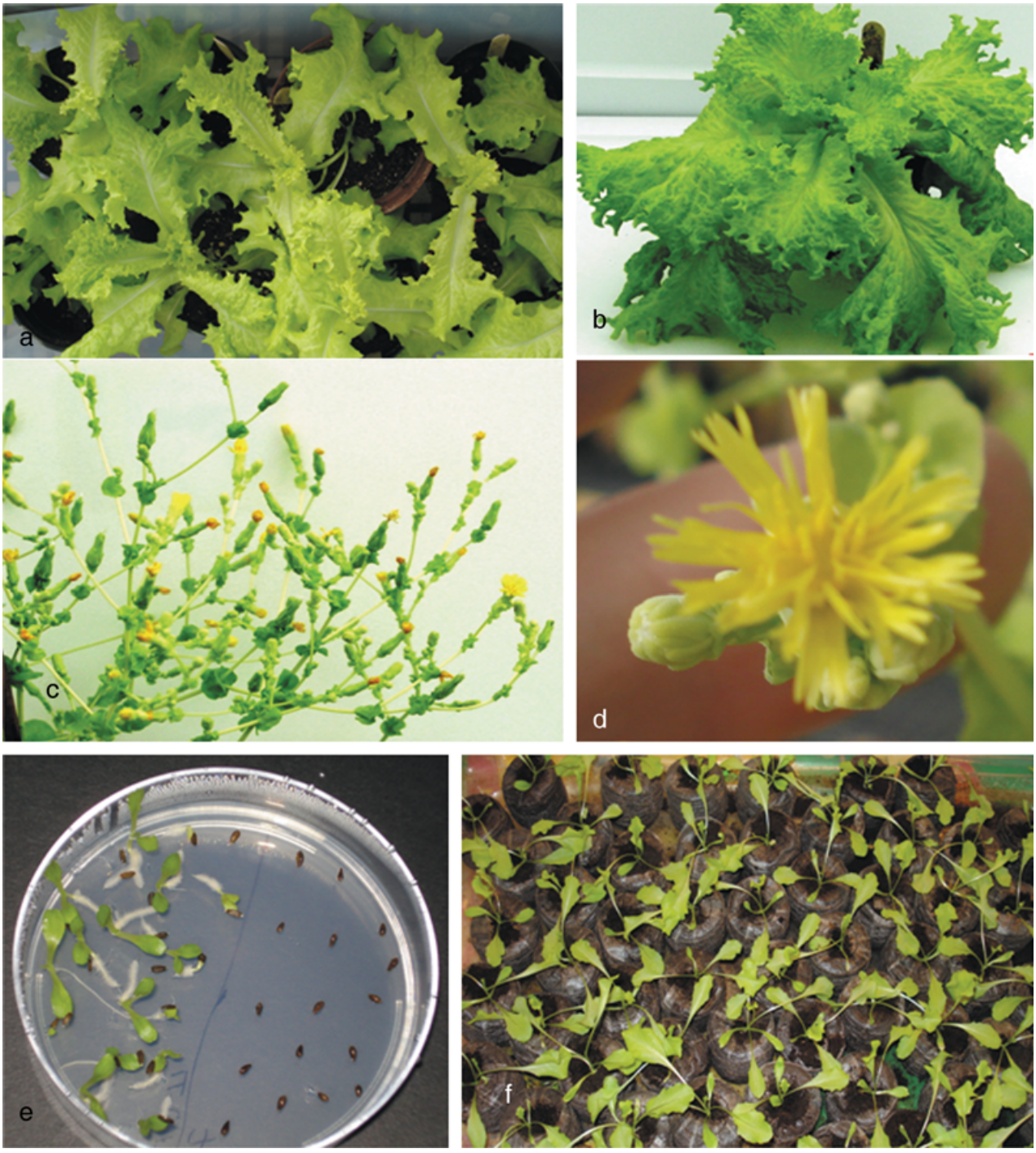

Figure 3 Production of transplastomic *Latuca sativa* and confirmation of maternal inheritance. (a) Lettuce plants propagated by rooting of nodal cuttings were transferred to soil in the glasshouse. Plants matured with no apparent aberrant phenotype (b) and produced normal inflorescences (c). Flowers heads opened (d), and seeds were harvested. T_1 seeds were plated on half-strength Murashige and Skoog (MS) medium containing 50 mg/L spectinomycin, together with wild-type seed (e). T_1 plants flourished and were transferred to the glasshouse (f).

Because cellular infiltration can lead to apoptosis, this can be used as a hallmark to study type 1 diabetes (Riedl and Shi, 2004). It was found that the β-cells from NOD mice treated with CTB-Pins rarely expressed caspase-3, suggesting that apoptosis was prevented in these cells. In the other experimental groups, even the very few remaining insulin-producing β-cells expressed activated caspase-3, suggesting that they were undergoing apoptosis (Figure 6).

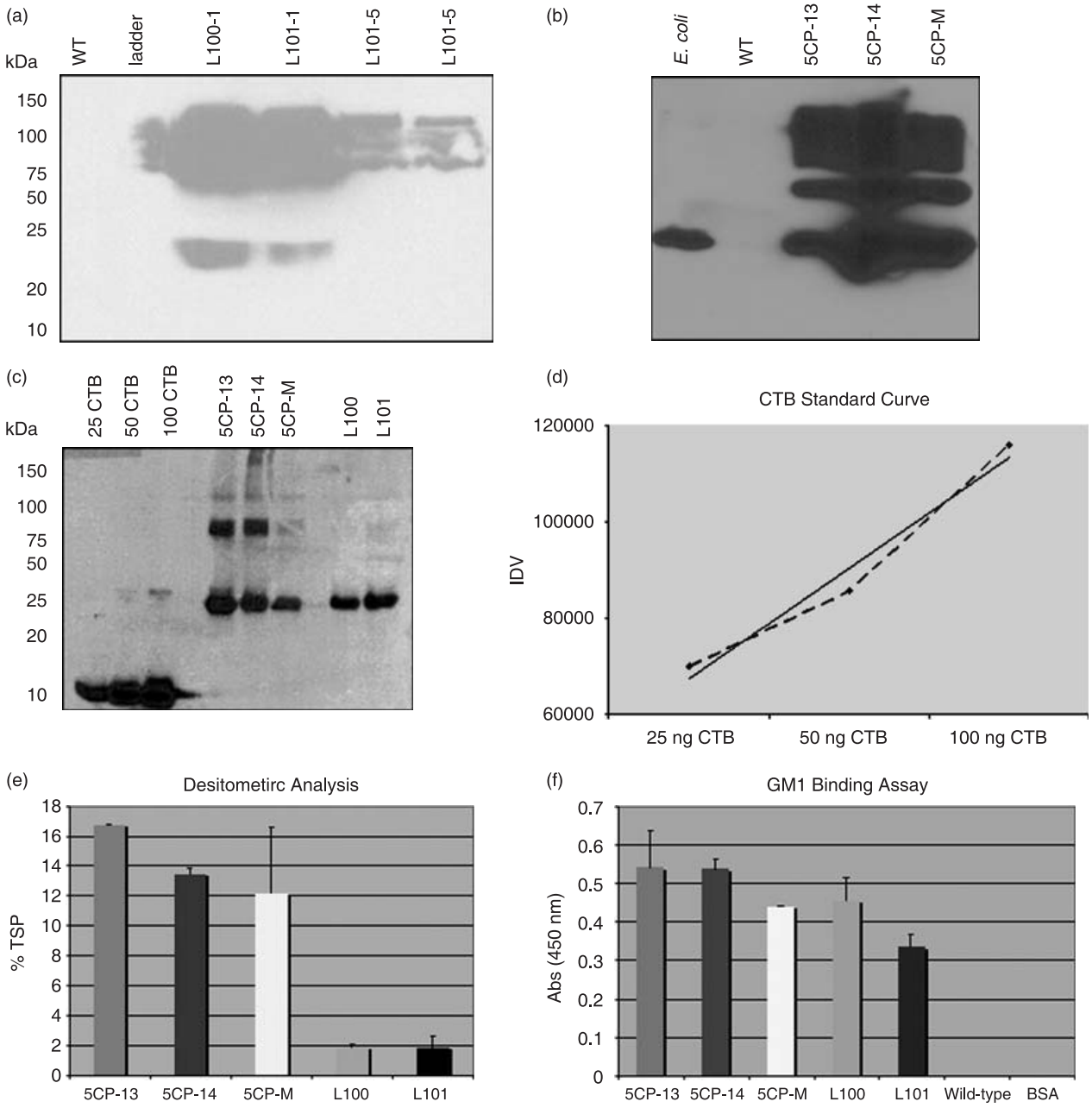

Figure 4 Protein analyses. Western blots prepared from wild-type (WT) and transplastomic lettuce and tobacco were probed with human proinsulin monoclonal antibody. (a) Total protein (~20 μg) extracted from lettuce leaves was loaded into wells for each sample. In lane 6, L101-5, ~10 μg of total soluble protein (TSP) was loaded. (b) Total protein (~10 μg) extracted from tobacco leaves was loaded into wells. (c) Cholera toxin B subunit–human proinsulin (CTB-Pins) in plant samples was detected by polyclonal anti-CTB and quantified by comparison with known quantities of CTB standard. Lanes 1–3, bacterial CTB (25, 50, 100 ng). Lanes 4–6, transplastomic tobacco lines 5CP-13, 5CP-14, 5CP-M (~6 μg of TSP in each lane). Lanes 7 and 8, transplastomic lettuce lines L-100 and L-101 (~36 μg TSP in each lane). (d) Plot of integrated density values (IDVs) for quantitative analysis from standard curve. Broken line, data points; full line, trend line. (e) Estimation of CTB-Pins in tobacco and lettuce leaves using spot densitometry CTB immunoblots. (f) Tobacco and lettuce transformants were assayed for GM_1 binding. 5CP, CTB-Pins tobacco; L101 and L101, CTB-Pins lettuce; BSA, negative control (bovine serum albumin).

Induction of T-helper 2 (Th2) response and production of immunosuppressive cytokines

Oral administration of CTB-Pins to NOD mice led to an increased recruitment of immunosuppressive cytokine-producing cells (lymphocytes) to the pancreas. A large number of interleukin-4 (IL-4) and interleukin-10 (IL-10)-producing cells were seen proximal to the pancreatic islets, which were recruited through the circulation. This process was supported by significant perivascular migration of

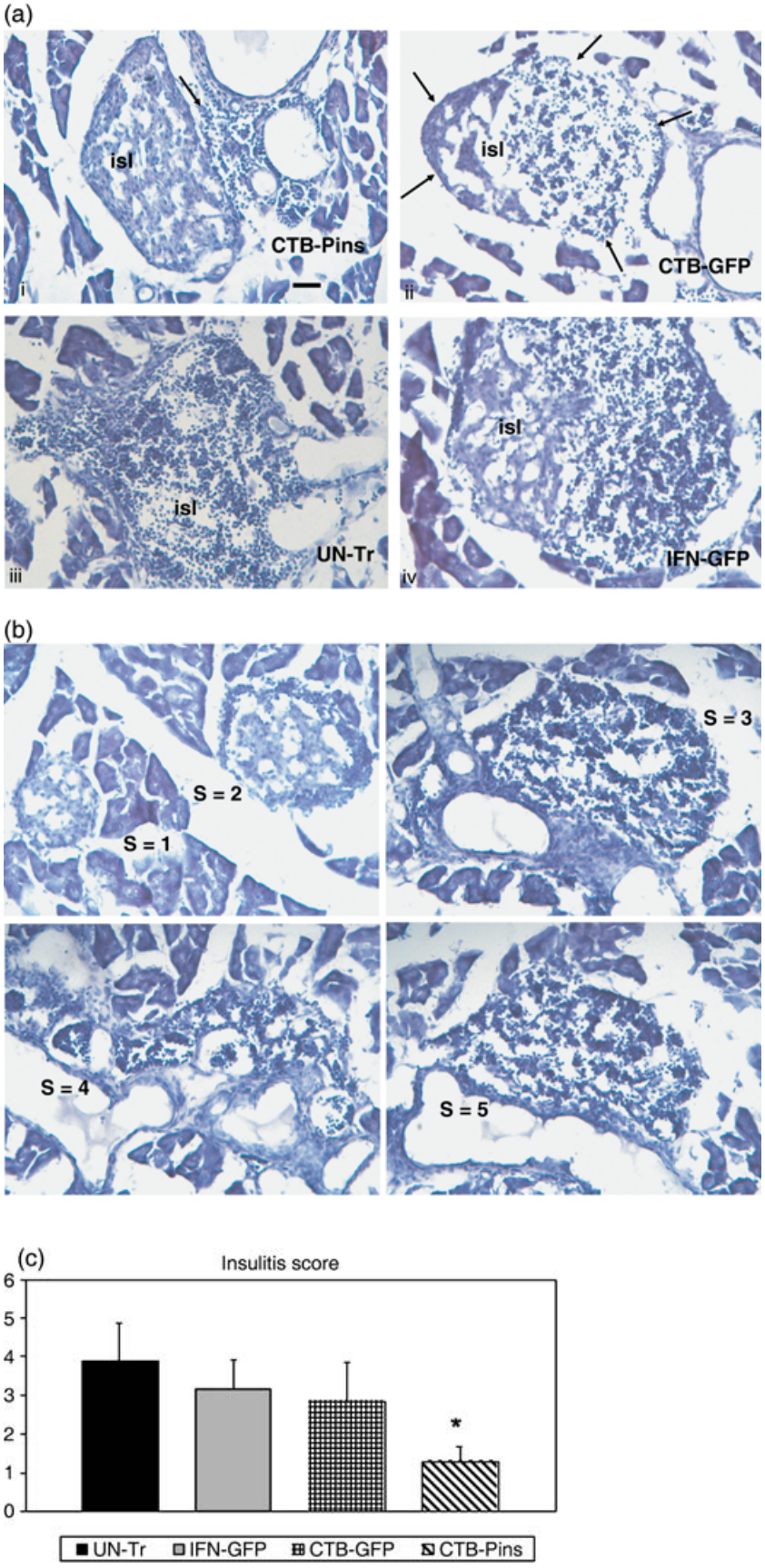

Figure 5 (a) Histochemical staining of pancreatic sections. (i) Haematoxylin and eosin staining of a section of the pancreas (showing an islet, isl) of a mouse receiving cholera toxin B subunit–human proinsulin (CTB-Pins) for 7 weeks (n = 7); scale bar, 50 μm. Lymphocytes are seen outside the islet (arrow). (ii) Arrows indicate the borders of an islet in the pancreas of a mouse receiving CTB–green fluorescent protein (CTB-GFP) (n = 5; control group). Blue dots show lymphocytic infiltration of the islet. (iii) A large islet with severe lymphocytic infiltration in a mouse receiving untransformed plant leaf material (UN-Tr; n = 3). (iv) Severe lymphocytic infiltration in a mouse receiving interferon–GFP (IFN-GFP) (n = 5). (b) Scoring (S) of the degree of insulitis according to the severity of the lymphocytic infiltration of the pancreatic Langerhans islets. Score 1, no or pre-islet infiltration; 2, minimal infiltration; 3, moderate infiltration; 4, severe infiltration; 5, more than 80% of the islets infiltrated. All sections were scored in a blind manner. (c) Graphic representation of insulitis scoring in untransformed, IFN-GFP, CTB-GFP and CTB-Pins plant-treated groups; bars represent standard deviation (*$P < 0.05$). n indicates the number of animals in each treatment group.

IL-4- and IL-10-expressing cells in the pancreases of CTB-Pins-treated NOD mice (Figure 7a,b).

Serum and intestinal immunoglobulin (Ig) levels following oral delivery of CTB-Pins

Serum and intestinal mucosal Ig levels were determined by ELISA using CTB as the capture antigen. Serum levels of IgG1 increased in NOD mice treated with CTB-Pins-expressing plant leaf material, relative to the control groups. Low serum IgG2a and mucosal IgA levels against CTB were observed in NOD mice treated with untransformed plant leaf material or plants expressing IFN-GFP or CTB-Pins (Figure 8a).

Blood and urine glucose levels of NOD mice treated with CTB-Pins

Blood and urine glucose levels of the treated NOD mice were measured twice, at weeks 6 and 7 of treatment (11- and 12-week-old mice). The blood glucose values of all groups in the study at both time points tested were below 200 mg/dL, and therefore were not considered as diabetic. This was not unforeseen, as NOD mice typically do not develop high blood glucose until 12–15 weeks of age (Arakawa *et al*., 1998). Although, at week 11, the CTB-Pins-treated group showed a blood glucose level as high as that of the control animals, interestingly, at week 12, the CTB-Pins-treated animals showed a significantly lower blood glucose level than that of the control groups. Urine glucose values were also lower in the CTB-Pins-treated group (Figure 8b).

Discussion

Oral tolerance, induced by the feeding of autoantigens, has been applied successfully as a therapeutic tool in experimental models of autoimmune diseases (Strobel and Mowat, 1998). The basic mechanism of oral tolerance in humans is currently a work in progress, and oral antigen administration regimens have resulted in limited success when applied to patients (Garside *et al*., 1999; Chaillous *et al*., 2000; Pozzilli *et al*., 2000). A possible explanation for this limited success may be that the doses of antigens administered orally to humans were low compared with those delivered to mice, considering the surface area of the intestinal absorptive epithelium (Pozzilli and Cavallo, 2000). In this case, CTB may serve as the necessary cofactor required to overcome the inefficient presentation of insulin to mucosal T-cells, resulting from the limited transport of native insulin across the epithelial layer. In order for oral tolerance to become a realistic therapy for human autoimmune diseases, adjuvants that possess the ability to enhance the tolerogenic potential of orally delivered antigens need to be identified. The coupling of

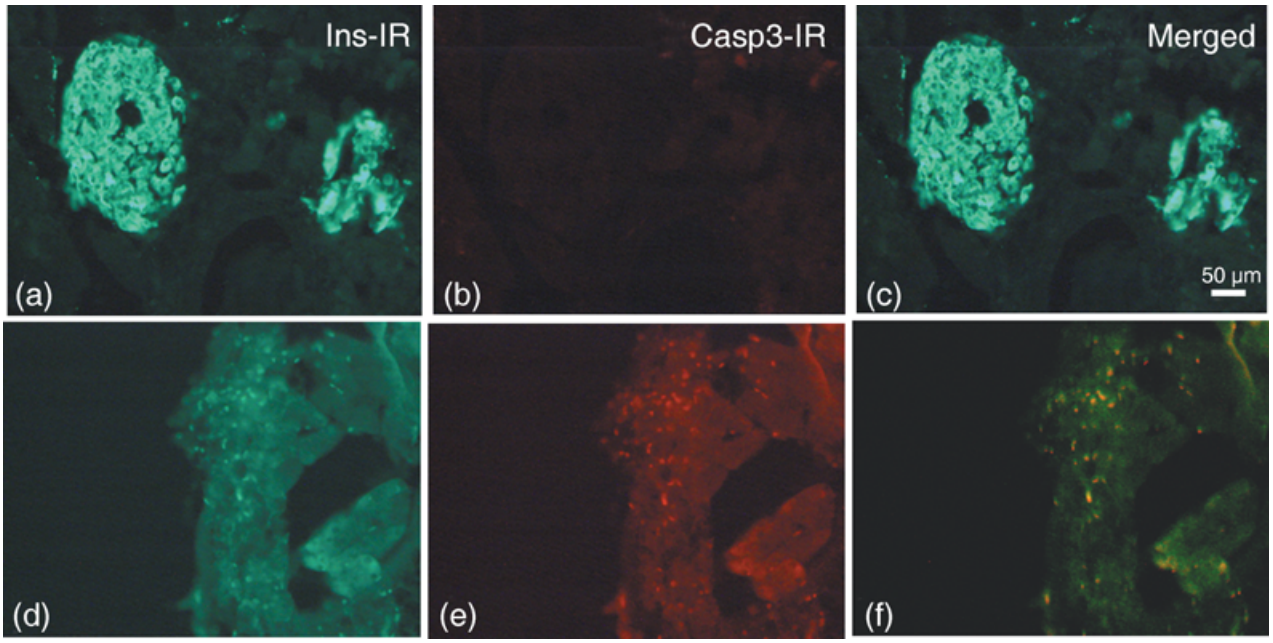

Figure 6 Assessment of insulin production and apoptosis in pancreatic β-cells. (a) Insulin immunoreactivity in Langerhans islets of a mouse receiving cholera toxin B subunit–human proinsulin (CTB-Pins). (b) Caspase-3 immunostaining in the same section is shown in the red channel. (c) Merged picture of (a) and (b). (d) A view of the pancreas showing the remnant of a large Langerhans islet in a mouse receiving untransformed plant leaf material. (e) Caspase-3 immunoreactivity in the same section taken in the red channel. (f) Merged picture of (d) and (e).

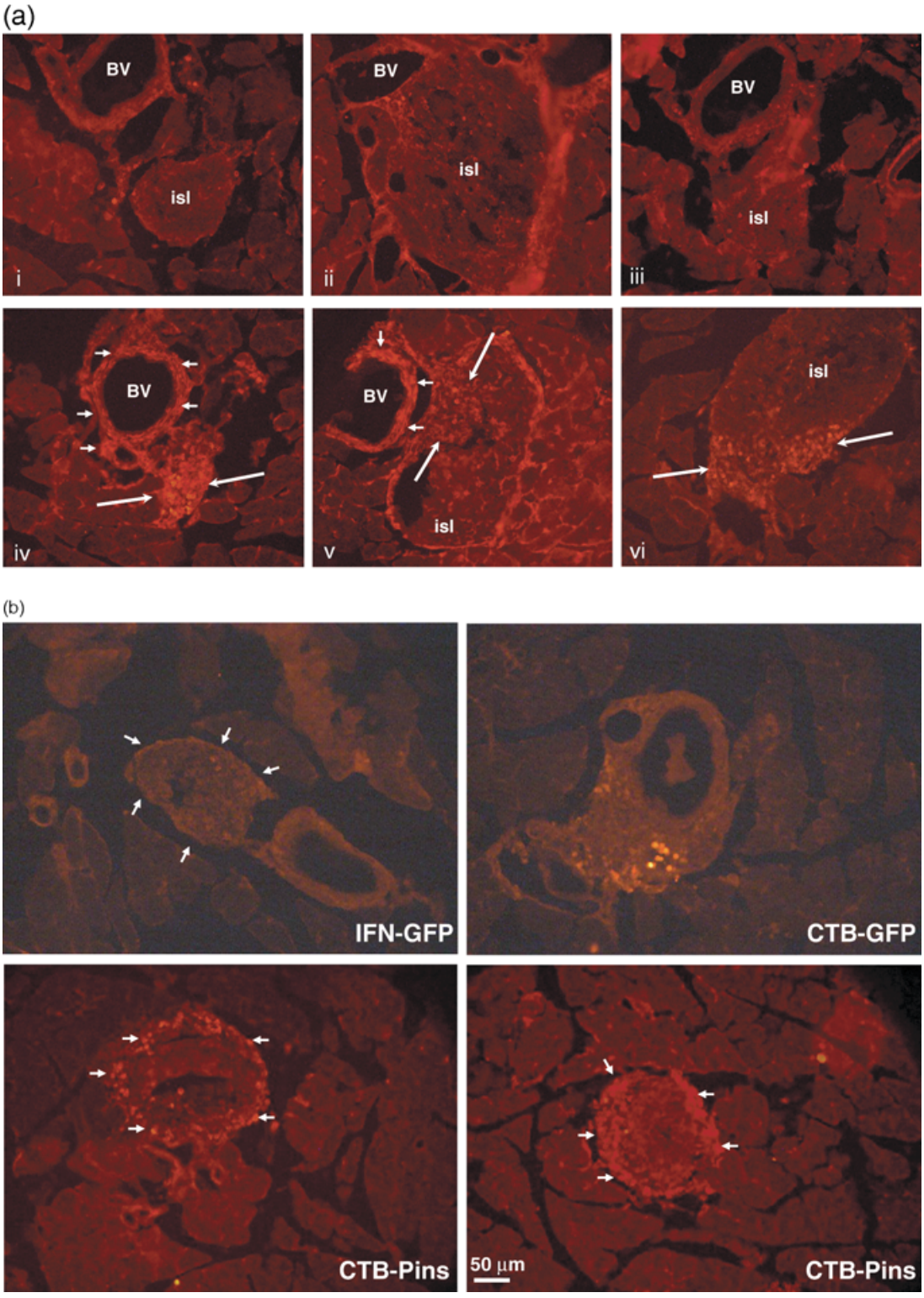

Figure 7 Immunostaining for cytokine production. (a) Interleukin-10 (IL-10) immunoreactivity in the pancreas of three mice administered untransformed plant leaf material (i–iii). Blood vessels (BV) and Langerhans islets (isl) are indicated. (iv–vi) Islets of mice receiving cholera toxin B subunit–human proinsulin (CTB-Pins). Small arrows indicate perivascular infiltration of IL-10-expressing lymphocytes. Large arrows indicate IL-10-positive lymphocytes inside or around the islets. (b) Interleukin-4 (IL-4) immunoreactivity in the pancreas of mice receiving interferon–green fluorescent protein (IFN-GFP), CTB-GFP or CTB-Pins plant leaf material. Small arrows indicate position of the islets.

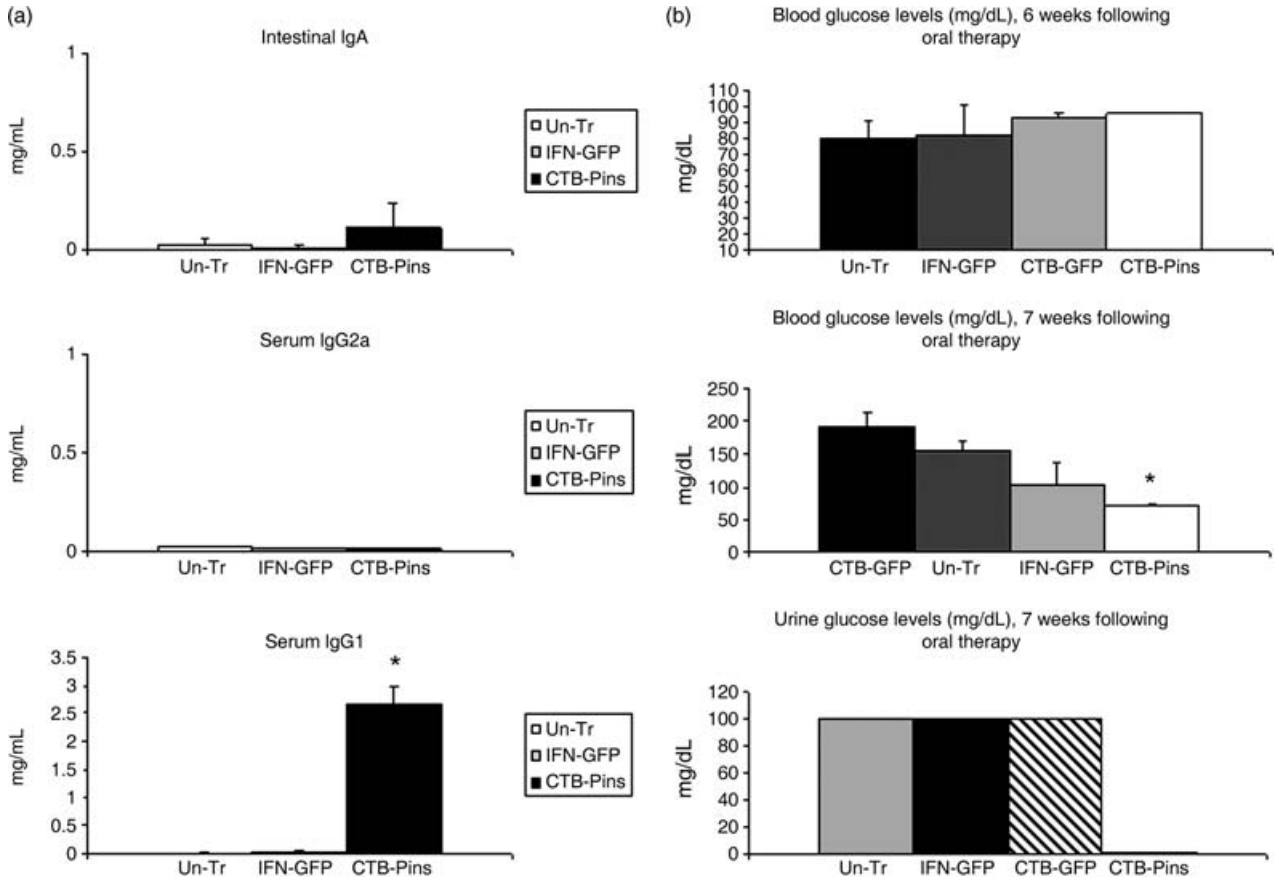

Figure 8 Determination of immunoglobulin (Ig) and glucose levels. (a) Serum levels of IgA, IgG2a and IgG1 in non-obese diabetic (NOD) mice receiving cholera toxin B subunit–human proinsulin (CTB-Pins)-expressing plant leaf material. Control groups receiving untransformed plant material (Un-Tr) or interferon–green fluorescent protein (IFN-GFP)-expressing leaf material are also shown. (b) Blood and urine glucose levels in various groups of NOD mice after oral administration of CTB-Pins (6 weeks post-treatment). Un-Tr, $n = 1$; IFN-GFP, $n = 1$; CTB-GFP, $n = 2$; CTB-Pins, $n = 2$. Using Student's t-test, P value is less than 0.05. Bars, standard deviation. n indicates the number of animals in each treatment group.

autoantigens – in this case, proinsulin – to non-toxic CTB dramatically increases their tolerogenic potential (Sun *et al*., 1994; Bergerot *et al*., 1997; Arakawa *et al*., 1998). This effect is mediated by the ability of CTB to act as a transmucosal carrier, although CTB may have a direct affect on the immune system (Burkart *et al*., 1999; Li and Fox, 1999). The current primary limitation in advancing this concept in clinical trials is the low levels of expression in transgenic plants (Bergerot *et al*., 1997). This limitation can be overcome by the hyper-expression of the CTB-Pins fusion protein in transgenic chloroplasts.

Previous studies expressing the CTB-Pins fusion protein in plants have been performed in potato (Arakawa *et al*., 1998). The expression level in nuclear transgenic potato tubers was 0.1% of TSP. This low expression level required the feeding of NOD mice with large amounts of fresh potatoes. In the present study, the CTB-Pins fusion protein accumulated in transplastomic tobacco to up to ~16% of TSP, 160-fold greater than that achieved in nuclear transgenic potatoes. NOD mice were given 8 mg of CTB-Pins tobacco leaf tissue (containing ~14 μg of the fusion protein) per week by gavage; this represents a 375-fold decrease in the amount of plant tissue administered compared with the 3 g per week used previously (Arakawa *et al*., 1998). The use of these small concentrated doses reduces the possibility of the potential confounding effects of leaf tissue, and eliminates the need to process or purify large quantities of plant material. Hyper-expression of CTB-Pins in plant plastids should make this fusion protein abundantly available for animal studies or human clinical trials.

Although lower levels of CTB-Pins accumulation were observed in transplastomic lettuce when compared with tobacco (approximately sixfold less in lettuce), the average value determined for lettuce (~1.8% of TSP) represents a level of protein sufficient to proceed with animal or preclinical studies. For example, in this study, using tobacco, we delivered approximately 14 μg of CTB-Pins to NOD mice; a comparable dose could be derived from 100 mg of fresh lettuce leaf,

a feasible quantity for weekly oral delivery. The difference observed between tobacco and lettuce may be attributed, in part, to the 5′ regulatory elements used in our study. Tobacco expression of CTB-Pins is driven by the endogenous *psbA* 5′ UTR, whereas lettuce expression is regulated by the inclusion of the translational control region of bacteriophage T7 gene 10. Previous studies have demonstrated that the level of foreign protein accumulation is lower when these translation elements are used to drive expression of the same gene, with *psbA* 5′ UTR being more efficient (Dhingra *et al.*, 2004). In addition, intrinsic variation in the nature of the leaves from tobacco and lettuce may influence the accumulation of foreign protein expressed in chloroplasts. We are developing new transformation constructs for lettuce for CTB-Pins expression which will employ lettuce endogenous translation elements, such as *psbA* 5′ UTR, to further increase the level of expression.

The oral administration of self-antigens, such as insulin, leads to their uptake by gut-associated lymphoid tissue (GALT), including intestinal mucosal M-cells, which pass the antigen to underlying antigen-presenting cells (Limaye *et al.*, 2006). This leads to the activation of T-cells and the induction of a Th2 cell response, which is characterized by the up-regulation of immunosuppressive cytokines (such as IL-10 and IL-4) and serum antibodies (such as IgG1, but not IgG2a) (Salmond *et al.*, 2002; Faria and Weiner, 2005). No significant increase in mucosal IgA was seen in our study in CTB-Pins-treated mice vs. the control groups. CTB-Pins-treated animals showed very high levels of IgG1, but not IgG2a, whereas the control groups showed no variation (Figure 8a). The serum IgG1 values of CTB-Pins-treated animals confirm the activated Th2 response, which is also supported by the histology, with less lymphocytic infiltration, the up-regulation of immunosuppressive cytokine levels in the tissues, and the trend in the blood and urine glucose levels (which were higher in the control groups than in the CTB-Pins-treated group). The presence of CTB in the intestine ensures effective receptor-mediated oral delivery of intact plant-derived fusion protein across the intestinal mucosa via the binding of CTB to the GM_1 ganglioside receptor and uptake by intestinal M-cells and enterocytes.

Taken together, the data presented here suggest that the suppression of insulitis is mediated by regulatory Th2 cells. As T-cell regulation plays a major role in mucosal immunity, the oral administration of an autoantigen can be used to treat autoimmune diseases in animal models by generating active T-cell suppression. Several autoimmune diseases and their antigens are known: multiple sclerosis (myelin basic protein and proteolipid protein), arthritis (type II collagen), uveitis (S-antigen and interphotoreceptor retinoid binding protein), myasthenia gravis (acetylcholine receptor) and thyroiditis (thyroglobin) (Hafler and Weiner, 1997). In the USA, 5.5 million people suffer from psoriasis, 3 million from Graves' disease, 2.5 million from rheumatoid arthritis, 2–5 million from vitiligo, 3.5 million from thyroiditis, 1–4 million from Sjogren's syndrome, 0.5 million from Crohn's disease and multiple sclerosis, 370 000 from type 1 diabetes, etc. This study opens up the possibility for new investigations on autoimmune diseases.

Five-week-old mice were used in this study to demonstrate the alleviation of symptomatic pancreatic insulitis and the preservation of insulin-producing β-cells, a condition that mimics human type 1 diabetes. Based on the success of the concept in older mice (Harrison *et al*., 1996), this strategy is likely to work not only prior to the onset of diabetes, but also at later stages of this autoimmune disease, and this will be explored in future experiments. One previous human clinical study on the oral delivery of insulin was unsuccessful (Skyler *et al*., 2005), because insulin was not protected from digestive enzymes and acid hydrolysis. In our study, however, insulin was protected by bioencapsulation within plant cells. On the basis of the results obtained in this study, human clinical trials have been initiated.

Experimental procedures

Vector construction

The human proinsulin gene was synthesized according to Prodromou and Pearl (1992). The PCR product was then cloned into the PCR 2.1 vector and the sequence was verified. The *psbA* promoter and 5′ UTR were amplified from the tobacco chloroplast genome, followed by subcloning and sequence verification. The promoter-5′UTR fragment was then spliced together with CTB and human proinsulin by overlap extension (Horton *et al*., 1989). The construct containing 5′UTR-CTB, a GPGP hinge region (introduced by mutagenesis to allow for the correct folding of each protein by reducing steric hindrance) and human proinsulin was designated as 5CP. Following *Sal*I/*Not*I digestion, the fusion gene was ligated into the pLD-ctv chloroplast transformation vector (Daniell *et al*., 1998, 2004b).

The pUC-based *L. sativa* long flanking plasmid (pLS-LF; Figure 2a) was constructed to integrate foreign genes into the intergenic spacer region between the *trnI* (Ile) and *trnA* (Ala) genes of the plastid genome inverted repeat region. Oligonucleotide primers were derived from regions of known conservation in the *N. tabacum* plastome, and carried end modifications for restriction enzyme sites for use in vector assembly. The full-length genes for plastid *trnI* (Ile) (position 101 979–102 998) and *trnA* (Ala) (position 103 063–103 949) were amplified and ligated into the pUC19 backbone. A unique *Pvu*II recognition site (position 103 002) between the two sequences facilitated the insertion of transformation cassettes. Long flanking sequences encoding the 3′ end of the 16S (100 876–101 979; 810 bp) and 5′ end of the 23S (104 102–105 075; 974 bp) ribosomal RNA subunits were amplified and cloned in kind. The numeric values

correspond to the positions in the *L. sativa* plastome (NC_007578). The transformation cassette (Figure 2a) was assembled in pZERO (Invitrogen, Carlsbad, CA, USA) and included the following published tobacco plastid regulatory sequence elements: ribosomal operon promoter (P*rrn*), *rps16* and *psbA*-3′ UTRs. The 5′ translation control region of bacteriophage T7 gene 10 was used to drive CTB-Pins expression and the *aadA* gene was included with a GGAGG ribosome binding site. The expression cassette was flanked with *Sna*B1 recognition sites. The *Pvu*II-digested pLS-LF was treated with alkaline phosphatase prior to ligation with the *Sna*B1-digested cassette. The recovered plasmids were digested with *Sac*I to determine the correct orientation of the inserted cassette in pLS-LF. All cloning steps were carried out in *Escherichia coli* according to the methods of Sambrook and Russel (2001).

Bombardment and selection of transgenic plants

The bombardment and regeneration of *N. tabacum* cv. Petit Havana transformants were carried out as described previously (Kumar and Daniell, 2004).

Seeds of *L. sativa* v. Simpson elite (New England Seed Co., Hartford, CT, USA) were surface sterilized in a 3% hypochlorite solution, rinsed three times in water and plated on Murashige and Skoog (MS) medium solidified with 5.8 g/L Phytablend® (Caisson, North Logan, UT, USA). Young, fully expanded leaves (~4 cm^2) were placed, adaxial side up, on antibiotic-free LR medium (Kanamoto *et al*., 2006). The leaves were bombarded with 0.6-μm gold particles (Bio-Rad, Hercules, CA, USA) coated with pLS-LF-*CTB-Pins* (Figure 2a), as described by Kumar and Daniell (2004), employing 900 psi rupture discs and a target distance of 6 cm. Samples were held in the dark at 25 °C for 2 days prior to the explan of 0.5-cm^2 pieces, adaxial side down, on to LR medium containing 50 mg/L spectinomycin dihydrochloride. Primary regenerants were screened by PCR for the transplastomic event, and positive shoots were subjected to an additional regeneration cycle on LR medium containing spectinomycin. Following the second regeneration, the shoots were rooted in half-strength MS medium containing 0.1 mg/L naphthaleneacetic acid (NAA) and 50 mg/L spectinomycin. The plants were propagated by the rooting of nodal sections in half-strength, hormone-free MS medium containing spectinomycin. Rooted cuttings were hardened in Jiffy® peat pots before transfer to the glasshouse for seed production. T_1 seeds (achene) were harvested when the pappus was present, and allowed to dry at 24 °C. Sterile seeds (100) were plated on MS medium containing 50 mg/L spectinomycin.

Southern blot analysis

Southern blot analysis was carried out essentially according to Kumar and Daniell (2004). Total tobacco DNA was digested with *Afl*III, separated on a 0.7% agarose gel at 45 V for 4 h, and then transferred to a nylon membrane. The pUC-CT vector DNA was digested with *Bam*HI and *Bgl*II to generate a 0.8-kb probe, which was used as a flanking probe, and pLD-*CTB-Pins* was digested with *Mfe*I and *Not*I to generate a 0.36-kb gene-specific probe. After labelling the probe with ^{32}P, hybridization of the membranes was performed using the QUICK-HYB hybridization solution and protocol (Stratagene, La Jolla, CA, USA).

For lettuce transformants, genomic DNA was digested to completion with *Bgl*II, separated on 0.7% Tris-acetate-EDTA (TAE)-agarose and transferred to nylon membranes. The plastid flanking sequence probe (1.3 kb) was amplified by PCR from lettuce genomic DNA. The PCR product was purified, and labelled probe was generated by incubation with ^{32}P and Ready-To-Go™ DNA Labelling Beads (-dCTP) (GE Healthcare, El Paso, TX, USA). Hybridization was carried out at 68 °C with washing at 37 °C and 60 °C. Radiolabelled blots were exposed to film at –80 °C for 16 h.

Western blot and densitometric analysis

TSP was isolated from rooted second regenerants of tobacco and lettuce. TSP from approximately 100 mg of leaf tissue (10 or 20 micrograms) was separated by sodium dodecylsulphate-polyacrylamide gel electrophoresis (SDS-PAGE) and transferred to nitrocellulose membranes for immunoblotting, according to Kumar and Daniell (2004). Anti-proinsulin monoclonal antibody (American Qualex, 1 : 20 000, San Clemente, CA, USA) and goat anti-mouse IgG conjugated to horseradish peroxidase (HRP) (American Qualex, 1 : 15 000) were used for the detection of the CTB-Pins protein. Immunoblotting with anti-CTB primary antibody (1 : 4000, Sigma, St. Louis, MO, USA) and HRP-conjugated donkey anti-rabbit secondary antibody (1 : 12 500, Biomeda, Foster City, CA, USA) was employed for spot densitometric analysis. For transplastomic lettuce samples, 36 μg of TSP was added to each well; for transplastomic tobacco samples, 6 μg of TSP was added to each well. The standards (CTB; Sigma) contained 25, 50 and 100 ng of purified bacterial CTB. A SuperSignal® West Pico HRP Substrate Kit (Pierce, Rockford, IL, USA) was used for autoradiographic detection. Following exposure to film, the blots were analysed for the presence of CTB-Pins using AlphaImager® and AlphaEase® FC software (Alpha Innotech, San Leandro, CA, USA), by comparison with known quantities of standard.

GM_1 binding assay

In order to test the ability of chloroplast-derived CTB-Pins to bind to the GM_1 receptor, a CTB–GM_1 binding assay was performed. Tobacco extracts were prepared in ELISA buffer and lettuce extracts were prepared in western extraction buffer. Ninety-six-well plates were incubated with monosialoganglioside-GM_1 (Sigma) (3.0 μg/mL in bicarbonate buffer) and bovine serum albumin (BSA) as a control, and incubated overnight at 4 °C. Following washing [three times each with 1X phosphate buffered saline with 0.05% (v/v) Tween 20 (PBST) and sterile water], the plate was blocked with 1X PBST (0.1% Tween 20) with 3% dry, non-fat milk (PTM) (tobacco) or 0.25% BSA in phosphate-buffered saline (PBS) (lettuce) for 1 h at 37 °C. CTB standards (Sigma) and soluble protein extracts were diluted in ELISA plant extraction buffer [without Tween-20 and phenyl methyl sulphonyl fluoride (PMSF)]. The standards and samples were then added in duplicate and incubated at 4 °C overnight. Rabbit anti-CTB primary antibody (1 : 8000 tobacco, 1 : 3000 lettuce; Sigma) and HRP-conjugated donkey anti-rabbit secondary antibody (1 : 25 000 tobacco and 1 : 12 500 lettuce; Biomeda) were used to detect the binding of CTB-Pins to the GM_1 receptor. The plate was washed thrice with both PBST and sterile water, and 100 μL of tetramethyl benzidine (TMB) soluble solution substrate (American Qualex) was added to the wells and incubated in the dark for 30 min. The reaction was stopped with 50 μL of 2 M H_2SO_4, and read on a plate reader (Dynex Technologies, Chantilly, VA, USA) at 450 nm.

Animal studies

Four-week-old, female, NOD mice were purchased from The Jackson Laboratory (Bar Harbor, ME, USA). Mice were kept in the UCF Wild Animal Facility under normal light/dark cycle conditions and had access to food and water *ad libitum*. The oral administration of CTB-Pins-expressing transgenic tobacco or control plant leaf material began when the animals were 5 weeks of age, to allow the mice 1 week to acclimatize to the facility. The mice were divided into the following groups: group 1 received untransformed plant leaf material (Un-Tr); group 2 received transgenic plant leaf expressing CTB conjugated to GFP (CTB-GFP); group 3 received transgenic plant leaf expressing IFN conjugated to GFP (IFN-GFP); and group 4 received CTB-Pins-expressing transgenic plant leaf. Each group contained five animals, except for the CTB-Pins group, which contained seven. The mice were given 14 μg of the specified ground tobacco leaf material diluted in 200 μL of PBS (0.1 M) by careful gavage into the hypopharynx once a week for 7 weeks. For preparation of the gavage material, leaves were frozen and ground in liquid nitrogen. For oral delivery, 8 mg of the CTB-Pins-expressing plant leaf material contained 14 μg of the CTB-Pins protein. For the untransformed leaf material, 8 mg of the ground plant material was given to mimic a similar oral dose to the CTB-Pins group. The CTB-GFP and IFN-GFP levels were similar to that of CTB-Pins. The animals were sacrificed at 12 weeks of age, the pancreas and other tissues were collected, and both blood and urine glucose levels were measured.

Histochemistry for lymphocytic infiltration and insulitis

Following the 7 weeks of treatment, the mice were sacrificed and perfused transcardially with 10 mL of PBS, followed by 50 mL of 4% paraformaldehyde (PFA) in 0.1 M PBS. Part of the pancreas was saved before fixation and freshly frozen in Tissue Tec freezing medium (Vector Laboratories, Burlingame, CA, USA). The remaining pancreas was removed, postfixed overnight in 4% PFA, and cryoprotected by serially passing through 10%, 20% and 30% sucrose solutions in PBS. The pancreatic tissue was then immersed in Tissue Tec freezing medium and frozen (Samsam *et al*., 2003) in liquid nitrogen-cooled isomethylbutane (isopathane, Sigma). Frozen sections of the pancreas, 10 μm thick, were prepared using a cryostat. Pancreas cryosections were stained with haematoxylin and eosin, dehydrated in serial graded alcohol solutions, and the slides were covered.

Insulitis levels were measured using the extent of lymphocyte infiltration of the islets of Langerhans. At least 50 sections per animal were scored. The degree of insulitis was scored on a scale of 1–5, where score 1 denotes a normal islet with no sign of T-cell infiltration, and score 5 indicates maximal infiltration and the development of insulitis.

Immunohistochemistry for insulin, caspase-3, IL-4 and IL-10

Immunohistochemistry for the localization of insulin, caspase-3 (a final molecule of apoptosis) and the immunosuppressive cytokines IL-4 and IL-10 was performed on pancreas cryosections. Sections were blocked with 10% BSA containing 0.3% Triton-X100.

Polyclonal guinea pig anti-insulin, polyclonal rabbit anticaspase-3, and rat monoclonal anti-IL-4 and anti-IL-10 primary antibodies (Invitrogen) were diluted at a concentration of 1 : 300 in 1% BSA in PBS containing 0.3% Triton-X. Fluorescent conjugated secondary antibodies were goat anti-guinea pig Alexa Fluor 488 (green), goat anti-rabbit Alexa Fluor 555 (red) and goat anti-rat Alexa Fluor 555 (red; Invitrogen).

Antibody titre

Serum and intestinal antibodies were assayed for the presence of anti-CTB antibodies using colorimetric ELISA methods. Following sacrifice and prior to transcardial perfusion, 200–300 μL of blood was collected from the retro-bulbar vein. Serum was extracted after 15 min of centrifugation at 3000 × ***g*** at 4 °C for antibody titre. Ninety-six-well plates were coated with CTB (Sigma). Serial dilutions of serum or supernatants of faecal pellets (five pellets per sample were weighed and diluted in 0.1 M PBS), collected from the different animal groups, were added to the coated plate wells. For standard values (isotype control) and determination of antibody level, the standard columns of the plate were coated with serial dilutions of purified mouse IgG1 or IgG2a, or IgA protein (BD Pharmingen, Franklin Lakes, NJ, USA), and ELISA was performed. Secondary antibodies were HRP-conjugated anti-mouse IgG2a, IgG1 and IgA antibodies (BD Pharmingen) at a concentration of 1 : 3000 in PBS containing 0.1% Tween-20 and 3% milk powder. The plates were washed with 200 μL of PBS, and the substrate TMB was added to the wells and incubated in the dark at 37 °C for 20 min. The reaction was stopped by adding 50 μL of H_2SO_4, and the plates were read on a plate reader (Dynex Technologies) at 450 nm.

Blood and urine glucose levels

Blood and urine glucose levels were measured for two consecutive weeks (11 and 12 weeks of age, or 6 and 7 weeks following the oral delivery of various plant leaf material). Urine glucose levels were measured using urinary glucose test strips (Clinistix and Diastix, Bayer, Wayne, NJ, USA), and blood glucose was measured using a blood glucose analyser (Boehringer Mannheim, Indianapolis, IN, USA) with blood obtained from either the tail vein or the retro-bulbar vein (at week 12, before sacrifice). Blood glucose levels above 250 mg/dL were considered to be diabetic (Arakawa *et al*., 1998). The first blood sample was taken from a small cut on the tail; one drop of blood was placed on the test strips provided by the manufacturer which were fed to the glucose analyser. The second blood sample was taken from the retro-bulbar vein after deep (lethal) inhalation anaesthesia with isoflurane before transcardial perfusion of 4% PFA. Urine samples were collected by pressing on the supra-pubic area and squeezing one to two drops of urine on to the urine strips provided by the manufacturer.

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